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Liposome-based synthetic long peptide vaccines for cancer immunotherapy

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Chapter 4

Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: a comparative study of cationic liposomes and PLGA nanoparticles

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Abstract

Nanoparticulate formulations for synthetic long peptide (SLP)-cancer vaccines as alternative to clinically used Montanide ISA 51- and squalene-based emulsions are investigated in this study. SLPs were loaded into TLR ligand-adjuvanted cationic liposomes and PLGA nanoparticles (NPs) to potentially induce cell-mediated immune responses. The liposomal and PLGA NP formulations were successfully loaded with up to four different compounds and were able to enhance antigen uptake by dendritic cells (DCs) and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice with the different formulations showed that the SLP-loaded cationic liposomes were the most efficient for the induction of functional antigen-specific T cells *in vivo*, followed by PLGA NPs which were as potent as or even more than the Montanide and squalene emulsions. Moreover, after transfer of antigen-specific target cells in immunised mice, liposomes induced the highest *in vivo* killing capacity. These findings, considering also the inadequate safety profile of the currently clinically used adjuvant Montanide ISA-51, make these two particulate, biodegradable delivery systems promising candidates as delivery platforms for SLP-based immunotherapy of cancer.

Keywords: cellular immune response, synthetic long peptides, TLR ligands, cationic liposomes, PLGA nanoparticles

Introduction

Peptide-based vaccine formulations offer several advantages over protein-based vaccines, as peptides can be easily synthesized and characterized, and are generally more stable [1] and better processed [2] than whole proteins. Synthetic peptides derived from tumour- associated antigens (TAAs) have attracted considerable interest as a basis for cancer vaccines, and vaccination with synthetic long peptides (SLPs), containing all the CTL and T_H epitopes of a TAA, has been applied in mouse models with superior efficacy to protein antigen [2] or minimal MHC class I restricted epitopes [3,4]. In contrast to short peptides, SLPs cannot bind directly to MHC molecules, but have to be taken up and processed by DCs like regular pathogens, inducing a stronger immune response, owing to the activation of both $CD4^+$ and $CD8^+$ T cells [4-6]. However, peptides alone are poorly immunogenic and need to be combined with adjuvants such as immune modulators and/or delivery systems in order to properly activate the innate and adaptive arms of the immune system [1].

Over the past few years, delivery systems that elicit strong immune responses, such as nano-emulsions and particulate delivery systems have been extensively studied. These include MF59 (Novartis) and AS03™ (GlaxoSmithKline), squalene-based oil-in-water emulsions, which have been approved in Europe for use in the Fluad® and Pandemrix™ influenza vaccines, respectively [7]. Despite the efficacy of these emulsions as influenza vaccine adjuvants, and though some degree of T_H1 responses have been observed, still they lack the ability to stimulate strong T cell responses [8]. Montanide (ISA-51, Seppic) water-in-oil (w/o) emulsions have shown to elicit CTL responses in clinical studies, and have been applied to formulate SLPs in several clinical therapeutic cancer vaccination trials [9-15]. However, the use of Montanide has some important limitations, such as non-biodegradability, limited long-term stability, poorly defined release properties, suboptimal efficacy, and in some cases induction of local adverse side effects [16,17]. Therefore, alternative delivery systems for SLP-based vaccines are highly needed.

Studies have shown that peptide-based vaccines may benefit from particulate delivery systems that mimic the size and structure of a pathogen, facilitating uptake by DCs and increasing cross-presentation of the peptide [18-20]. Importantly, they can harbour multiple vaccine components and be actively or passively targeted to DCs, also shaping the induced immune response via specific receptors, such as toll-like receptors, by incorporation of TLR ligands. Among several particulate delivery systems, both liposomes and polymeric particles have been widely studied.

We have previously studied the application of poly-(lactic-co-glycolic-acid) (PLGA) NPs [21] and cationic liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [22] for the encapsulation of a 24-amino acid-long SLP (referred to as OVA24) harbouring the CTL epitope SIINFEKL of ovalbumin (OVA). Encapsulation of this SLP in PLGA NPs led to a significant enhancement of MHC class I antigen presentation and $CD8^+$ T cell activation compared to free SLP *in vitro* [21]. The SLP-specific $CD8^+$ T cell frequency induced *in vivo* by a liposomal SLP formulation containing poly(I:C) showed a 25-fold increase compared to poly(I:C)-adjuvanted free SLP. Furthermore, intradermal immunisation of mice with SLP-liposomes combined with poly(I:C) led to a strong cytotoxic activity, in contrast to vaccination with a mixture of free SLP and poly(I:C) [22].

In this study, considering the different physicochemical properties that cationic liposomes and PLGA NPs have, we further investigated the potential of both systems in a direct comparative study. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the T helper (T_H, OVA17) epitopes of OVA together with poly(I:C) and $Pam3CSK_4$, a TLR3 and TLR2/1 ligand, respectively, in comparison to the clinically used adjuvants Montanide ISA-51 and SWE, a squalene oil-in-water emulsion. OVA24/OVA17-loaded PLGA NPs and liposomes with or without the TLR ligands were characterized for particle size, zeta-potential, and for peptide and TLR loading efficiencies. The obtained formulations were assessed *in vitro* and *in vivo* for their potency to induce $CD8^+$ and $CD4^+$ T cell immune responses. The observed T cell immune responses induced by our particulate formulations were superior to the ones observed with the emulsions (Montanide ISA-51 or SWE), with the liposomal formulation outperforming PLGA NPs. These findings reinforce that particulate systems are promising delivery vehicles for clinical application in cancer immunotherapy.

Materials and Methods

Materials

The ovalbumin-derived SLP OVA24 [DEVSGLEQLESIINFEKLAAAAAK], including the CTL epitope SIINFEKL, and the short peptide OVA8 [SIINFEKL] were produced and purified at the GMP facility of the Clinical Pharmacy and Toxicology Department at the Leiden University Medical Center [5]. The ovalbumin-derived SLP OVA17 [ISQAVHAAHAEINEAGR], including the helper T_H-epitope AAHAEINEA, was produced in the Immunohematology and Blood Transfusion Department of the Leiden University Medical Centre. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and the TLR ligands (poly(I:C) and Pam3CSK$_4$) with their labelled analogues (rhodamine and fluorescein) were obtained from InvivoGen (Toulouse, France). Resomer® RG 502H was purchased from Boehringer Ingelheim (Ingelheim, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, the Netherlands), PVA 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Sodium hydroxide was purchased from Boom (Meppel, Netherlands). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/ml penicillin and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionized water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Montanide ISA-51 was purchased from Seppic SA (Paris, France). Squalene oil-in-water emulsion (SWE) contained 3.9% (w/v) squalene, 0.5% (w/v) Tween 80 and 0.5% (w/v) Span 85 in 10 mM citrate buffer pH 6.5 and it was manufactured by the Vaccine Formulation Laboratory of the University of Lausanne. Phosphate buffer was composed of 7.7 mM $Na_2HPO_4.2H_2O$ and 2.3 mM $NaH_2PO_4.2H_2O$, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, which was used for all the *in vitro* and *in vivo* assays was purchased from B. Braun (Meslungen, Germany). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

Mice

Female C57BL/6 (H-2^b) mice were purchased from Charles River (L'Arbresle, France) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Centre animal facility and used at 8-14 weeks of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe.

Liposome preparation

Cationic liposomes loaded with SLPs were prepared by using the thin film dehydration-rehydration method, as described previously [22]. Briefly, DOTAP and DOPC (1:1 molar ratio) in chloroform were mixed in a round-bottomed flask to reach a concentration of 10 mg total lipid per ml of final liposome dispersion. The formed dry film was rehydrated with 2 ml of 1 mg/ml OVA24 and/or OVA17 in ACN/H_2O 1:1 (v/v); for the liposomes loaded with both OVA24 and OVA17, the aqueous solution of the SLPs was first adjusted to pH 8.5. For poly(I:C)-loaded liposomes, the ligand (including 0.5% fluorescently-labelled equivalent) in a total concentration of 200 μg/ml was added dropwise to the dispersion, while for the Pam3CSK_4-loaded liposomes, the TLR ligand was dissolved in chloroform together with the lipids, before the dry film formation. After the lipid film hydration, the liposome dispersion was snap-frozen in liquid nitrogen, followed by overnight freeze-drying. Dehydrated-rehydrated liposomes were generated by gradually adding 10 mM PB, pH 7.4, to the freeze-dried lipid cake. Liposomes were down-sized by high-pressure extrusion at room temperature using a Lipex extruder (Northern Lipids Inc., Canada) and concentration of peptide-loaded liposomes was performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa) as described previously [22].

PLGA NPs preparation

Nanoparticles loaded with OVA24 and/or OVA17 and/or TLRLs were prepared by using a double emulsion with solvent evaporation method [21]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane, with or without 0.25 mg Pam3CSK_4 (and 0.1% Pam3CSK_4 Rhodamine-labelled), were emulsified under sonication (30 s, 20 W) with 1.4 mg OVA24, 1 mg OVA17, 1 mg poly(I:C) (and 0.1% Poly(I:C) fluorescein-labelled, dissolved in 50% ACN in 0.25 mM NaOH + 400 μL HEPES pH 8.0). To this first emulsion (w1/o), 2 ml of 1% PVA solution were added immediately, and the mixture was emulsified again by sonication (30 s, 20 W), creating a double emulsion (w1/o/w2). The emulsion was then added dropwise to 10 ml of extraction medium (0.3% w/v PVA)

previously heated to 40°C under agitation, to allow quick solvent evaporation, while stirring, which was continued for 1 h. The particles were then collected by centrifugation for 15 min at 15000 g at 10°C, washed, resuspended in deionized water, aliquoted and freeze-dried at -55°C in a Christ Alpha 1-2 freeze-dryer (Osterode am Harz, Germany) for 12 hours.

Liposome and PLGA NP characterization

Average diameter (Z_{ave}) and polydispersity index (PDI) of the formulations were determined by dynamic light scattering (DLS) using a Zetasizer (NanoZS, Malvern Ltd. UK). The same instrument was used for zeta-potential determination by laser Doppler electrophoresis. For these measurements, liposome samples were diluted 100-fold in PB, pH 7.4. Peptide loading efficiency was determined by extracting OVA24 and OVA17 from the liposomes using a modified Bligh-Dyer method and applying a UPLC method, as described previously [22].

For the PLGA NPs, the Z-average size, polydispersity index and zeta-potential were measured after the freeze-dried NP were resuspended in 1 mM HEPES pH 7.4 to a final concentration of 10 mg PLGA/ml. Peptides' loading efficiency was determined by measuring the peptide content of digested particles by reversed phase HPLC, as described previously [21].

Loading efficiency of poly(I:C) and $Pam3CSK_4$ was calculated by fluorescence detected with an Infinite® M 1000 Pro (Tecan, Switzerland) microplate reader (excitation/emission wavelengths: 492 nm/518 nm for fluorescein and 549 nm/566 nm for rhodamine).

Montanide ISA-51 and SWE emulsions preparation and characterization

Preparation of Montanide ISA-51 emulsion was performed by diluting the SLPs, $Pam3CSK_4$ and poly(I:C) in PBS and mixing with Montanide ISA-51 water-in- oil for 30 min in a 1:1 (v/v) ratio, using a vortex mixer. The squalene-based formulation, SWE, was prepared as previously described [23,24]. For the loading of the SLPs and adjuvants, the SWE was diluted with vaccine medium to 2% (v/v) squalene to the same ratio and mixed gently for 10s prior to immunisation.

In vitro MHC class I antigen presentation

Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with SLP-loaded formulations or plain SLP (with or without TLR

ligands) in PBS at different concentrations. After 2.5 hours T cell reporter hybridoma B3Z cells ($50x10^5$/well) were added and the mixture was incubated overnight at 37°C. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and colour conversion was measured by detecting absorbance at 590 nm.

Immunisation of mice

Mice were immunized with SLP-loaded formulations or free peptides, OVA24 and OVA17 (with or without TLR ligands), by subcutaneous injection in the tail base. All formulations were prepared on the day of injection. Vaccination dose was based on the OVA24 SLP concentration, 1 nmol (2.5 µg) of peptide in a total volume of 100 µl, and immunisations were performed on day 0 (prime immunisation) and on day 14 (boost injection). Vaccinations with adjuvanted vaccines included a dose of 0.5 – 1.0 µg of a TLR ligand. During the *in vivo* studies, blood samples were obtained from the tail vein at different time points.

Analysis of antigen-specific $CD8^+$ and $CD4^+$ T cell responses by flow cytometry

Staining of the cell surface was performed on blood samples after red blood cell lysis. Cells were stained in staining buffer for 30 min with allophycocyanin labelled tetramer-OVA8 (TM-SIINFEKL) and fluorescently labelled antibodies specific for mouse CD3 (BD Biosciences), CD4, CD8 (eBiosciences). 7-Aminoactinomycin D (Life Technologies) was used for the exclusion of dead cells.

Overnight intracellular cytokine analysis of PBMCs was performed after incubating the cells with 2 µM of OVA8 and 2 µM of OVA17, in presence of brefeldin A (7.5 µg/ml) (BD Biosciences, Breda, the Netherlands). After the overnight cells incubation the assay was developed as previously described [22].

In vivo cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were lysed and split into two equal parts. Cells were labelled with CFSE and adoptively transferred intravenously in previously immunized recipient C57BL/6 mice in a volume of 200 µl in PBS as described [22]. Two days after the cell transfer (day 24), mice were sacrificed, spleens were isolated and single cell suspensions were analysed by flow cytometry. Specific killing (SK) was calculated according to the following equation:

$$\mathrm{SK} = \left\{ 1 - \frac{\left[\frac{\mathrm{CFSE\ high}}{\mathrm{CFSE\ low}}\ \mathrm{vaccinated\ mice}\right]}{\left[\frac{\mathrm{CFSE\ high}}{\mathrm{CFSE\ low}}\ \mathrm{naive\ mice}\right]} \right\} \mathrm{x}\ 100\ \%$$

Results

Characterisation of adjuvanted SLP-loaded liposomes and PLGA NPs

We have previously shown that effective tumour vaccines require the inclusion of both CTL and T_H epitopes [5,25]. In this study, a 24-mer SLP covering a CTL epitope and a 17-mer covering a T helper epitope of ovalbumin, designated as OVA24 and OVA17, respectively, were used as model antigens to study the effect of co-encapsulation of these SLP adjuvanted with poly(I:C) and $Pam3CSK_4$ (TLR3- and TLR2-ligands, respectively) in liposomes and PLGA nanoparticles (NPs). Our main objective was the direct comparison of the immunogenicity of the different systems, relative to that of a squalene- oil-in-water emulsion (SWE) used at preclinical stage studies and the clinically Phase I/II used Montanide ISA-51 water-in-oil emulsion.

SLP-loaded liposomes were prepared by adjusting the dehydration-rehydration method so that the highest loading of both SLPs in the liposomes was achieved. As we reported for OVA24 [22], entrapment of OVA17 in the liposomes seems to be also dependent on electrostatic interactions between the cationic liposomes and the peptide. OVA17 has an isoelectric point (pI) of 6.0 (higher than the pI of OVA24, ca. 4.25) and the highest loading efficiency for both SLPs, when separately loaded or co-encapsulated in liposomes, was achieved at a relatively high pH of about 8.5, i.e., above their isoelectric point (pI). At this pH, both peptides are expected to be strongly negatively charged, favouring the electrostatic interactions with the positively charged liposomes and yielding the highest loading efficiency for both SLPs.

The obtained SLP-liposomes had an average diameter that ranged from 147 nm (only SLPs-loaded liposomes) to 180 nm (OVA24/Pam/poly(I:C)-loaded liposomes). Liposomes with poly(I:C) or poly(I:C) combined with $Pam3CSK_4$ were larger and less monodisperse (PDI > 0.2). The positive zeta-potential was about 26 mV, independent of the formulation (Table I). The loading efficiency of OVA24 and OVA17 in unadjuvanted liposomes was about 46 % and 20%, respectively, and practically independent of the co-encapsulation of poly(I:C) and $Pam3CSK_4$ (Table I), suggesting that there is no competition between the TLRLs and the two peptides.

PLGA NPs were prepared by a double emulsion with solvent evaporation method described by Silva et al. [21]. Irrespective of the type of the loading, PLGA NPs were negatively charged, with a zeta-potential ranging from -11 to -14 mV (Table I), with a final particle size varying from 260 to 360 nm and a PDI below 0.3. The loading efficiency varied between 21 - 30% for OVA24, 31 – 36% for OVA17, 65 – 75% for $Pam3CSK_4$ and

53 – 73% for poly(I:C). We have previously shown the importance of the pH of the inner aqueous phase for the effective encapsulation of peptides in PLGA NPs and how crucial low burst release is in order to induce a cellular response [21]. Therefore, both OVA24 and OVA17 SLP were formulated at pH 8.0 to achieve optimal encapsulation and a relatively low burst release of circa 30% within 24 h (data not shown).

Table I: Physicochemical properties of SLP (+TLR ligand)-loaded formulation

				LE (%)			
	Z_{ave} diameter (nm)	PDI	ZP (mV)	OVA24	OVA17	TLR ligand Pam3CSK$_4$	TLR ligand Poly(I:C)
OVA24/Pam/poly(I:C) liposomes	180 ±10	0.29 ±0.03	26 ±3	56 ±5	NA	40 ±5	60 ±6
OVA24/OVA17 liposomes	147 ±10	0.21 ±0.02	25 ±2	46 ±7	20 ±5	*NA*	
OVA24/OVA17/Pam/poly(I:C) liposomes	175 ±20	0.27 ±0.05	26 ±2	46 ±5	20 ±5	40 ±5	55 ±8
OVA24/OVA17/poly(I:C) liposomes	173 ±20	0.28 ±0.05	27 ±2	42 ±10	20 ±3	*NA*	60 ±4
OVA24/Pam/poly(I:C) PLGA	260 ±19	0.19 ±0.02	-14 ±1	26 ±3	*NA*	67 ±7	60 ±2
OVA24/OVA17 PLGA	355 ±13	0.24±0.02	-14 ±1	30 ±10	36 ±6	*NA*	
OVA24/OVA17/Pam/poly(I:C) PLGA	357 ±45	0.22 ±0.02	-14 ±2	21 ±8	31 ±5	65 ±7	73 ±5
OVA24/OVA17/poly(I:C) PLGA	350 ±23	0.22 ±0.02	-14 ±3	29 ±10	35 ±6	NA	70 ±8
OVA24/OVA17/Pam/poly(I:C) ISA-51	500 ±20	0.25 ±0.04	42 ±5	*NA*	*NA*	*NA*	
OVA24/OVA17/Pam/poly(I:C) squalene (SWE)	136 ±15	0.15 ±0.00	-20 ±3	*NA*	*NA*	*NA*	

Data are averages ± SD of at least 3 independent batches
Z_{ave} average particle diameter in nanometres, *PDI* polydispersity index, *ZP* zeta-potential, *LE* loading efficiency, *NA* not applicable

Altogether, the data presented in table I shows that the model SLPs, OVA24 and OVA17, can be efficiently and reproducibly loaded in liposomes and PLGA NPs, also in presence of up to two TLR ligands.

In vitro SLP cross-presentation

The immunogenicity of the SLP formulations was first tested *in vitro*, based on their efficiency to activate immature DCs and cross-present the processed SIINFEKL-harbouring peptide (OVA24) to the $CD8^+$ antigen-specific (B3Z) T cells leading to their activation. Immature DCs were incubated with liposomes and PLGA NPs including both SLPs and both TLR ligands. Improved concentration-dependent activation of $CD8^+$ T cells was observed when particles were used compared to free peptides (OVA24+OVA17+Pam+poly(I:C)). Moreover, although PLGA NP plots consistently show

a larger dose-response effect, liposomes seem to be more effective in antigen presentation at lower concentrations (Figure 1).

Furthermore, the B3Z assay suggests efficient processing and presentation of OVA24 SLP by the DCs, irrespective of the presence of the OVA17 SLP (Figure 1). In addition to that, the incorporation of the TLR ligands poly(I:C) and Pam3CSK$_4$ did not significantly affect the *in vitro* T cell activation by the SLP-loaded particle formulations, as expected (Figure 1).

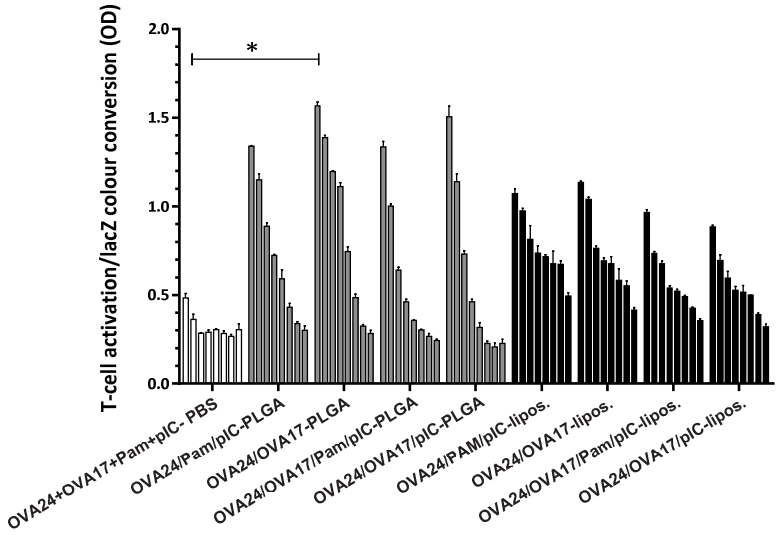

Figure 1: *In vitro* OVA-specific CD8$^+$ T cell priming. SIINFEKL-specific B3Z T cell activated after overnight co-culture with DCs and titrated amounts (4.0-0.0625 µM, 2-fold dilution series) of SLPs/TLR ligands-loaded formulations. Graphs show T cell activation based on the optical density (OD) at 590 nm after colour conversion of cell lysates after addition of CPRG (lacZ substrate). *p<0.05, evaluated with one-way ANOVA with Tukey's post hoc test and calculated with Mann-Whitney non-parametric test. Pam = Pam3CSK$_4$; pIC = poly(I:C)

In vivo induction of CD8$^+$ T cells

We evaluated the capacity of our particulate formulations to induce cell-mediated immune responses *in vivo* after subcutaneous vaccination at the tail base of mice, an administration route that appeared to enhance drainage to the lymphatic system in a more efficient way compared to subcutaneous delivery in the flank (unpublished data). The *in vivo* vaccine potency of liposomes and PLGA NPs was directly compared with that of the Montanide ISA-51 and SWE adjuvants.

In blood of mice immunized twice with SLP(s)-loaded liposome or PLGA NP formulations, on day 21 a high percentage of antigen-specific $CD8^+$ T cells (above 1% of the total $CD8^+$ T cell population) was detected in most groups, whereas in the Montanide ISA-51 or SWE groups this percentage remained below 0.5% (Figure 2A). In detail, it appeared that the OVA24/Pam/poly(I:C)-liposomes were more potent than the OVA24/Pam/poly(I:C)-PLGA NPs, expanding the percentages of antigen-specific $CD8^+$ T cells to about 6% and 2%, respectively.

Incorporation of the T_H epitope-SLP (OVA17) did not seem to improve the $CD8^+$ T cell proliferation induced by the PLGA NP formulations, while the addition of at least one TLR ligand, such as poly(I:C), seemed to be essential for T cell activation.

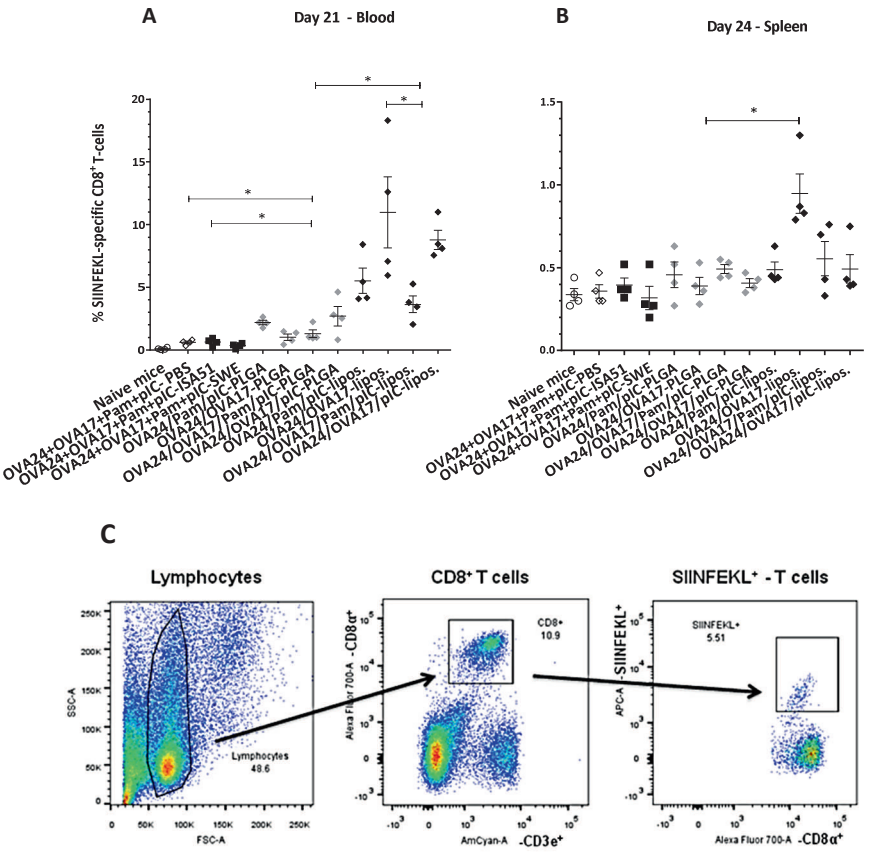

Figure 2: OVA24-specific $CD8^+$ T cell responses in blood (Day 21) and in splenocytes (Day 24) following subcutaneous immunisation with 1 nmol of SLPs on day 0 and 14 (A and B). Representative gating strategy for detection of SIINFEKL-specific $CD8^+$ T cells by flow cytometry using specific MHC class I tetramers (C). * $p<0.05$, ** $p<0.01$ calculated with by one-tailed Mann-Whitney test. ISA51= Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

On the other hand, incorporation of the two SLPs in the liposomes (OVA24+OVA17) seemed to be sufficient for the induction of the highest frequency of $CD8^+$ T cells (> 5 %) in blood of vaccinated mice, compared to all other liposomal groups (Figure 2A). Incorporation of poly(I:C) to the formulation with the two SLPs did not seem to further increase the number of the induced T cells in blood, while co-encapsulation of the lipophilic $Pam3CSK_4$ seemed to result in lower numbers of antigen-specific T cells. On day 24 in the analysed splenocytes of vaccinated mice, the *ex vivo* (non-restimulated) T cell responses in most groups were comparably low, except for the OVA24/OVA17-liposomes group, which showed a $CD8^+$ T cell frequency of about 1% (Figure 2B).

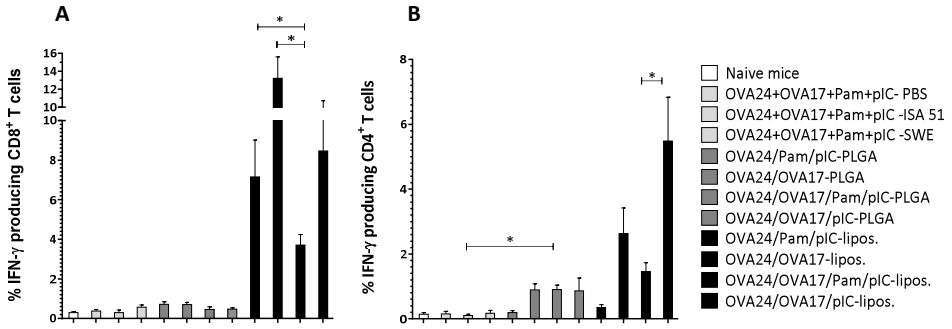

Figure 3: Intracellular cytokine analysis after immunization with OVA SLP-loaded particles. Blood of immunized mice at day 21 was stimulated *ex vivo* overnight with the minimal SIINFEKL epitope and OVA17. Plots show percentages (+SEM) of $CD8^+$ (A) and $CD4^+$ (B) T cells producing interferon gamma (IFN-γ). * $p<0.05$, calculated with by one-tailed Mann-Whitney test. ISA51 = Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

In vivo cytokine and cytotoxicity induction

Next to the T cell expansion potency of the vaccine formulations also the cytokine-producing functionality of the induced T cells was analysed. Thus, blood samples from the immunized mice were re-stimulated with MHC class I and class II binding peptides (short SIINFEKL and OVA17) *ex vivo* and the percentages of $CD8^+$ and $CD4^+$ T cells producing interferon gamma (IFN-γ) or IFN-γ and TNF-α simultaneously (data not shown), were assessed by intracellular cytokine staining.

In re-stimulated blood samples from mice immunized with PLGA NPs loaded with OVA24, $Pam3CSK_4$ and poly(I:C) (OVA24/Pam/polyI:C-PLGA) a higher percentage of cytokine-producing $CD8^+$ T cells was detected (ca. 0.7%) in comparison to the Montanide ISA-51 formulation (ca. 0.3%). This frequency did not increase after loading of OVA17 in the formulation, while all PLGA NPs showed a stronger induction of IFN-γ-producing

$CD4^+$ T cells (ca. 0.8 %) in comparison with the Montanide ISA-51 and SWE emulsions (ca. 0.1 %) (Figure 3B).

Mice vaccinated with liposomes loaded with both SLPs, with or without the two TLR ligands (OVA24/OVA17/Pam/poly(I:C)-liposomes and OVA24/OVA17-liposomes, respectively) showed an at least eight-fold higher efficiency to induce functional IFN-γ-producing $CD8^+$ T cells, as compared to the Montanide ISA-51- and SWE formulations (Figure 3A). As also observed in the SIINFEKL-specific $CD8^+$ T cell induction analysis (Figure 2), incorporation of poly(I:C) and $Pam3CSK_4$ into liposomes did not increase the number of induced T cells, although still much higher than the free SLP and TLR ligands. Although $Pam3CK_4$ is known to improve SLP vaccination by itself [25], its incorporation into the liposomes seems to have a inhibitory effect on the vaccination efficacy of the cationic liposomes, suggesting a possible change in the liposomes properties which influences the particles targeting.

Altogether, all liposomal formulations appeared to be significantly more efficient than any of the PLGA formulation tested (Figure 3). Furthermore, apart from $CD8^+$, functional $CD4^+$ T cells were detected in all groups treated with OVA17 (+poly(I:C))-containing liposomal formulations as well as PLGA NPs, indicating that OVA17 retains its functionality when co-encapsulated with OVA24 (Figure 3) and pointing out the importance of poly(I:C) presence for induction of antigen-specific $CD4^+$ T cells.

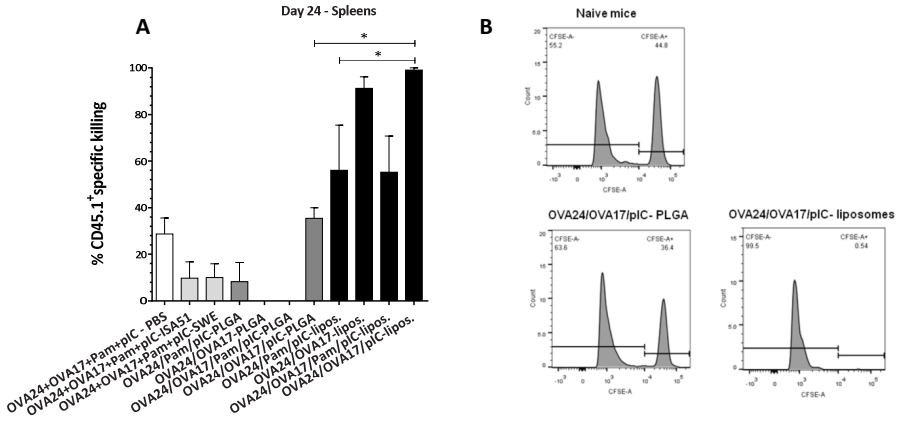

Figure 4: *In vivo* cytotoxicity against SIINFEKL-presenting target cells. The mean percentage of the killing activity of each SLP formulation is presented based on the frequency of the transferred $CD45.1^+$ cells that could be detected in splenocytes of mice immunized twice with the different formulations containing 1 nmol of OVA24. Bar graphs show the mean percentages (+SEM) of killed cells on day 24 (A). Representative histograms of CSFE-labelled positive target cells (right peak=SLP pulsed and left peak=negative control) (B). * $p<0.05$ calculated with by one-tailed Mann-Whitney test. ISA 51= Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

Finally, to test the effectiveness of our particulate vaccines to induce a strong functional cell-mediated immune response, the cytotoxic capacity of the induced $CD8^+$ T cells was tested in an *in vivo* cytotoxicity immunoassay, based on the killing of SIINFEKL-loaded target cells which were injected intravenously on day 22 in immunised mice (Figure 4).

Incorporation of poly(I:C) into PLGA NPs including both SLPs (and not $Pam3CSK_4$) appeared to be crucial for activation of $CD8^+$ T cells with a cytotoxic activity up to 40%, four times as high compared to the emulsions (Figure 4). In splenocytes of all mice vaccinated with liposomes a high killing capacity above 60% was detected. Mice immunised with liposomes containing both SLPs (OVA24/OVA17-liposomes) showed maximal *in vivo* cytotoxicity with or without poly(I:C).

According to the functional data of both the intracellular cytokine production and cytotoxicity assay, we can conclude that liposomal formulations loaded with the SLPs are superior to both PLGA NPs and Montanide ISA-51 or squalene-SLP-contained emulsions, when a rather low dose of SLPs (≤ 1 µg of SLP) is used like in the current study. Moreover, it appeared that the inclusion of an adjuvant ($Pam3CSK_4$ or poly(I:C)) in the liposomal formulation might not be necessary for the priming of a T cell immune response, since solely the presence of the T_H epitope seems to facilitate a T_H1-type pro-inflammatory immune reaction which is essential for effective therapeutic vaccines, to support a robust and long-lasting anti-tumour $CD8^+$ T cell response.

Discussion

There is a growing interest in therapeutic vaccination against cancer. The identification of tumour associated antigens (TAAs) has allowed the development of novel therapeutic strategies resulting in tumour regression. However, fine-tuned vaccines are required to reach the optimal potency and eventually replace the suboptimal formulations currently used. A major advantage of using particulate vaccines, such as liposomes and PLGA NPs, is their modularity. By tuning their physicochemical properties like size, charge and hydrophobicity, not only the stability of the particles and their antigen release pattern can be improved, but also the amount of antigen uptake by DCs, and priming of DCs towards cross-presentation, resulting in a more effective T_H1 type CTL response, required for cancer immunotherapy.

In this study we showed the successful co-encapsulation of two SLPs (OVA24 and OVA17) and two TLR ligands (poly(I:C) and $Pam3CSK_4$) in two different particulate delivery systems. The SLP-loaded and adjuvanted PLGA NPs and cationic liposomes were rather different in size and surface charge since PLGA NPs are negatively charged and liposomes are positively charged, while their different chemical nature is expected to influence the SLPs/TLR ligands localization and *in vivo* release profiles. Considering all possible differences, their potential for the induction of a cell-mediated immunity was investigated in comparison with two other systems, Montanide ISA-51, a water-in-oil emulsion, and SWE, a squalene-based oil-in-water emulsion.

As we showed here, co-delivery of a T_H antigen with a CTL epitope increases the expression of effector cytokines. This is most likely due to the stimulation of the MHC molecules displayed by DCs: CD40/CD40L ligation plays an important role in the activation of DCs and is a crucial stimulus for $CD4^+$ T_H-based $CD8^+$ T cell priming [26,27]. In addition, incorporation of a TLR ligand to the formulation, such as poly(I:C), can promote the active targeting and shape the immune response towards a more CTL--restricted manner. It has been shown that poly(I:C) stimulation of $CD8a^+$ DCs in mice led to successful cross-priming of $CD8^+$ T cells [28], improved the survival of $CD4^+$ T cells and produced functional $CD8^+$ memory even in the absence of $CD4^+$ T cells [29]. These findings are in line with our data which presented an improved functionality of the activated T cells by formulations where poly(I:C) is present. In contrast, inclusion of $Pam3CSK_4$ in the formulations did not further improve the induced immune response. This may be due to its lipophilic nature, resulting in localization of the $Pam3CSK_4$ lipopeptide in the lipid bilayer of the liposomes, or the polymeric matrix of the PLGA NPs, which might negatively affect the functionality of the TLR ligand, and thereby its

immunogenicity. With regard to the PLGA NPs, unadjuvanted PLGA NPs are considered to have very low immune-stimulating properties [30,31]. The formulation including both SLPs and poly(I:C), but not Pam3CSK$_4$, was the most promising for the induction of functional and cytotoxic T cells. Although the effect of Pam3CSK$_4$ on the induced immune response in PLGA NPs was not as pronounced as in liposomes, a negative influence by its presence was also observed, suggesting a change in the PLGA NPs which leads to a less efficient formulation or a different targeting that does not favour the induction of a T cell-based immune response. With respect to that, although we did not measure antibody levels in Pam3CSK$_4$-immunised mice, the trigger of an anti-bacterial humoral response rather than a cellular one could be possible through Pam3CSK$_4$–mediated signalling of the extracellular TLR1/2.

In this study we showed that for SLPs-based vaccines, cationic liposomes appeared to be the most potent delivery system, followed by PLGA NPs. It has been reported by van Duikeren et al. [32] that the efficacy of a therapeutic vaccine formulation can be predicted based not only by the levels of specific T cells, but also on the activation status of the antigen-specific T cell responses that are elicited when applied in non-tumour bearing mice. According to this, high frequencies of antigen-specific CD8$^+$ T cells with functional properties, such as production of effector cytokines combined with a strong killing capacity, can correlate with the therapeutic efficacy of the vaccine formulation that initiates this response. In our study, the SLP-loaded PLGA-based formulations induce relative high numbers of SIINFEKL-specific T cells, but these CD8$^+$ T cells are not strong IFN-γ –producers and they present a poor killing activity against target cells. In contrast, the strong killing capacity (> 80%) that the two liposomal formulations showed, combined with the high levels (5-10 %) of specific T cells induced can be used as an indication of their efficacy as therapeutic cancer vaccines to achieve protection.

It is known that the size of particulate adjuvants is crucial for their adjuvant activity and the immunogenicity difference observed in this study between liposomes and PLGA NPs may be partly attributed to the different particle sizes. In general, APCs are able to take up different particles ranging from the size of viruses (20-300 nm), bacteria (0.5-2 µm) up to whole cells, which can be bigger than 10 µm. Upon vaccination, small particles (10-150 nm) can easily penetrate the extra-cellular matrix (ECM) and be quickly transported into the lymph nodes [33] where they will interact with lymph node resident DCs [34]. Moreover, particulate systems with a size below 200 nm, such as the liposomes used in this study, will likely be taken up by DCs more efficiently than bigger particles [35], which are more prone to be recognized by macrophages and other scavenger immune cells, leading to a poorer T cell activation capacity [31,36]. In addition, it was suggested that

smaller particles (20-150 nm) are naturally taken up by endocytosis, resulting in cellular immune response, while larger particles, such as PLGA NPs used here, are more likely to be phagocytosed, leading to a predominantly humoral immune response [35].

Furthermore, the particles' size combined with the most efficient administration route has also an impact on antigen uptake and therefore can affect the efficacy of immunotherapy. Particles larger than 150 nm cannot be efficiently transported via the lymphatic system as mentioned above, and a percentage of the administered particles will be trapped in the tissue, creating a depot. As a general rule, the preferred route should be able to efficiently deliver the antigen to APCs and initiate a rapid T cell-based immunity. Although there are many administration routes nowadays under investigation [37], the most commonly used route remains the subcutaneous one. Subcutaneous vaccination at the tail base of mice, as the administration route selected for the *in vivo* studies presented in this study, appeared to enhance the drainage to the lymphatic system in a faster and more efficient way compared to subcutaneous delivery in the flank. With regard to Montanide ISA-51, although the exact adjuvant mechanism is not well understood, it is believed that such a water-in-oil emulsion creates a sustained release from the local antigen depot. However, a longer retention time of larger particles does not necessarily correlate with better antigen uptake and a stronger induced immune response [38]. Considering that, our data suggest that the internalization of particles, such as PLGA or liposomes, may be more important for the induction of an efficient cellular immune response than the formation of a depot.

Surface charge is another variable parameter between the particulate systems investigated in this study, since the PLGA NPs were negatively charged and the liposomes positively charged. However, it is not clear whether positively, negatively or neutrally charged particles are the best choice to induce effective T_H1-type cellular immune response. Investigations so far have revealed contradictive results. For instance, anionic PLGA NPs induced antibody responses as well as strong CTL responses and T_H1-biased cytokine release in mice and macaques [39]. Anionic PLGA particles also showed a higher accumulation in the lymph node compared to PEGylated particles of the same size [40]. However, anionic liposomes interacted with a limited fraction of human and murine DC populations [41], setting cationic liposomal formulations in favour. This outcome is in line with published research data where cationic liposomes were considered to be a very potent choice for immunotherapy [42] and we have recently reported efficient cellular response induction in vivo with DOTAP based cationic liposomes carrying synthetic long peptides antigens [22]. It was suggested that the positive charge promotes electrostatic interactions with the negatively charged cell

surface, thus interacting more efficiently with DCs and other APCs [41]. Positively charged liposomes showed the induction of a superior antigen specific cellular immune response, in comparison with negative or neutral liposomes [43]. Moreover, cationic particles are thought to have an adjuvant effect themselves [44]. It was found that cationic liposomes, but not anionic or neutral ones, can stimulate the expression of DC maturation markers such as CD80 and CD86, depending on the lipid structure, but did not lead to pro-inflammatory cytokine- or enhanced NF-κB expression, suggesting that they act independently of this pathway [45]. The partially contradicting results could be due to different formulation procedures, immunisation protocols and antigen characteristics used in reported studies. The latter may result in a charge-dependent entrapment efficiency and antigen release pattern.

The reason why liposomes appeared to be a more potent delivery system for the induction of a T cell-based immune response upon therapeutic vaccination than PLGA NPs is not yet fully clear. The nanoparticulate systems differ in several aspects, such as size, zeta potential and molecular composition, all of which may contribute to differences in biodistribution as well as activation and maturation of DCs, in favour of cationic liposomes. Once the particles have reached their APC (DCs) target, the intracellular processing of the antigen is equally crucial for the efficiency of the cross-presentation, as the ability of DCs to cross-present exogenous antigens is not intrinsic, but can depend on the route of antigen uptake and the following processed antigen's accumulation in the endolytic compartments [46]. Mechanistic studies of biodistribution, cellular uptake, DC activation and intracellular antigen processing are needed in order to gain insight into important parameters underlying the performance of both SLP-loaded formulations, cationic liposomes and PLGA NPs, which could help to further improve their properties for effective immunotherapy of cancer.

Conclusions

In this study we successfully co-encapsulated four compounds (two antigenic SLPs and two TLR ligands) in two different delivery systems, cationic DOTAP-based liposomes and PLGA NPs. In a direct comparative study, we compared the immunogenicity of the particulate formulations with that of two emulsion-based adjuvants, Montanide ISA-51 and squalene SWE. The capacity of the particulate systems to induce functional antigen-specific T cells was at least as good (PLGA NPs) or better (cationic liposomes) than that of the emulsion-based formulations. This, while also considering the unfavourable safety profile of the currently used adjuvant Montanide ISA-51, makes these particulate delivery systems attractive candidates as delivery platforms for SLP-based immunotherapy of cancer.

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